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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/767,749	01/28/2004	Ira Tabas	60921-A/JPW/AJM/MVM	6443

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EXAMINER	
BUNNER, BRIDGET E	
ART UNIT	PAPER NUMBER
1647	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	02/13/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Application No.	Applicant(s)	
	10/767,749	TABAS, IRA	
	Examiner	Art Unit	
	Bridget E. Bunner	1647	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *14 November 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,2,13,14,27 and 50-64* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,2,13,14,27 and 62-64* is/are rejected.

7)☒ Claim(s) *50-61* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *8/23/04 and 1/28/04* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☒ Other: *Appendices A and B*.

DETAILED ACTION

Status of Application, Amendments and/or Claims

The amendment of 14 November 2006 has been entered in full. Claims 1 and 10 are amended. Claims 50-64 are added. Claims 3-12, 15-26, and 28-49 are cancelled.

Election/Restrictions

Applicant's election with traverse of the species of amphiphilic compound (U18666A) in the reply filed on 14 November 2006 is acknowledged. As discussed in the previous Office Action of 09 August 2006, the species are independent or distinct because each of the compounds listed as (a)-(n) have different structural and functional characteristics. The species are independent or distinct because each requires separate, non-coextensive searches. Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141

The requirement is still deemed proper and is therefore made FINAL.

Claims are under consideration in the instant application.

Specification

1. The disclosure is objected to because of the following informalities:

1a. An updated status of the parent nonprovisional application should be included in the first sentence of the specification. For example, a statement reading "This invention is a continuation-in-part and claims priority to U.S. Serial No. 09/553,927, filed April 21, 2000, now abandoned..." should be entered.

1b. The reference cited on page 48, lines 15-16 (Yao et al.) should be updated.

Appropriate correction is required.

Claim Objections

2. Applicant is advised that should claim 1 be found allowable, claim 13 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

3. Applicant is advised that should claim 2 be found allowable, claim 14 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

4. Claims 50-61 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Claim Rejections - 35 USC § 112

5. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

6. Claims 1-2, 13-14, and 27 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for (1) a method for inhibiting macrophage death in a subject having, or at increased risk for developing cardiovascular disease comprising administering the

amphiphilic compound, 2β-(2-diethylaminoethoxy)-androstenone (U18666A); a method for inhibiting atherosclerotic lesional complications in a subject having, or at increased risk for developing cardiovascular disease comprising administering the amphiphilic compound, 2β-(2-diethylaminoethoxy)-androstenone (U18666A); and (3) a method for inhibiting atherosclerotic lesional necrosis in a subject having, or at increased risk for developing cardiovascular disease comprising administering the amphiphilic compound, 2β-(2-diethylaminoethoxy)-androstenone (U18666A) *does not reasonably provide enablement* for methods of administering all possible amphiphilic compounds, including a method for inhibiting necrosis, plaque rupture and/or superficial erosion. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The claims are directed to a method for inhibiting macrophage death in a subject having, or at increased risk for developing, cardiovascular disease which comprises administering to the subject an effective amount of an amphiphilic compound which inhibits the intracellular transport of cholesterol within cell, so as to thereby inhibit macrophage death in the subject. The claims also recite methods of inhibiting atherosclerotic lesional complications in a subject and for inhibiting necrosis, plaque rupture and/or superficial erosion in a subject by administering an effective amount of an amphiphilic compound.

The specification of the instant application teaches that LDL receptor knockout mice are fed a diet containing cholesterol and saturated fat for 12 weeks in the presence or absence of 0.75 mg/kg/d U18666A (pg 72, lines 20-30). The specification discloses that the U18666A treatment group exhibited a marked reduction in atherosclerotic lesion progression as measured by lesion

area, acellular area, and lipid core area (pg 73, lines 1-7; Figure 14A-E). However, the specification does not teach any methods or working examples to indicate that all possible amphiphilic compounds inhibit macrophage death, inhibit atherosclerotic lesional complications, or inhibit necrosis, plaque rupture, and superficial erosion in a subject having, or at increased risk for developing, cardiovascular disease. Undue experimentation would be required of the skilled artisan to administer all possible amphiphilic compounds to a subject and determine their effect *in vivo*. The specification of the instant application teaches that "'amphiphilic compounds' include, without limitation, compounds which inhibit cholesterol esterification (e.g., steroids such as progesterone), hydrophobic amines, phenothiazines, ionophores, cytochalasins, lysophosphatides such as lysophosphatidylcholine, lysophosphatidylserine and lysophosphatidylethanolamine, colchicine, nigericin, chloroquine, chlorpromazine, trifluoperazine, monensin and amphipathic amines such as imipramine and UI8666A" (pg 26, lines 29-33; pg 27, lines 1-5). However, the state of the art is such that the compounds listed by the specification are structurally and functionally diverse from one another. For example, phenothiazine is an anti-psychotic agent that has a three-ring structure in which two benzene rings are linked by a sulfur and a nitrogen atom (Goodman and Gilman's", 1993, New York: McGraw-Hill; Baldessarini, R.J., pages 386-397). Colchicine is an anti-inflammatory agent largely effective against gouty arthritis and is an alkaloid of *Colchicum autumnale* (Goodman and Gilman's", 1993, New York: McGraw-Hill; Insel, P.A., pages pg 674-676). Chloroquine is a type of 4-aminoquinoline that has been used to treat malaria, extraintestinal amebiasis, rheumatoid arthritis, discoid lupus erthyematosus, porphyria cutanea tarda, solar urticaria, and polymorphous light eruption (Goodman and Gilman's", 1993, New York: McGraw-Hill;

Webster, Jr., L.T., pages 978-983). Thus, one skilled in the art would not be able to predict that all amphiphilic compounds (except U18666A, as disclosed in the example of the instant specification) would be able to inhibit macrophage death, inhibit atherosclerotic lesional complications, or inhibit necrosis, plaque rupture, and/or superficial erosion in a subject.

Furthermore, there are no methods or working examples in the instant specification to indicate that any amphiphilic compound, *including U18666A*, is able to inhibit all forms/types of necrosis in all possible cells in a subject with cardiovascular disease (other than atherosclerotic lesional necrosis) (see for example, Appendices A and B attached to the instant Office Action; http://mozcom.com/~emcdvm/path02.html and http://www.fleshandbones.com/readingroom/pdf/852.pdf). There are also no methods or working examples in the instant specification to indicate that any amphiphilic compound, *including U18666A*, is able to inhibit plaque rupture or superficial erosion in a subject. Relevant literature teaches that one of the drawbacks of animal models of atherosclerosis is the lack of end-stage atherosclerosis with spontaneous plaque rupture that is characterized by an area of fibrous cap disruption, whereby the overlying thrombus is in continuity with the lipid core (Lutgens et al. Arterio Thromb Vasc Biol 23: 2123-2130, 2003; pg 2123, col 2, last paragraph). Lutgens et al. continue to disclose that "[a]lthough some features of plaque rupture occur spontaneously in genetically engineered mouse models of atherosclerosis, they should not be defined as plaque rupture according to the strict definition as proposed by Virmani et al. Mouse and rabbit models of acute plaque rupture resemble human ruptured lesions but require mechanical intervention or the use of vasoconstricting agents, which might not reflect pathogenesis in humans" (pg 2124, col 2, 2nd full paragraph). Lutgens et al. state that the

pathogenesis of plaque erosion is hardly known (pg 2123, col 2, top continuing paragraph). Additionally, the pathogenesis of plaque rupture involves many factors, not simply macrophage death. For example, Lutgens et al. teach that several regulators in the development of plaque rupture include matrix turnover, influx of inflammatory mediators, coaguluation modulators, and systemic factors (altered blood rheology, increased coagulability, increased systemic inflammation, recurrent infections) (pg 2125-2126). Therefore, a large quantity of experimentation would be required of the skilled artisan to administer all possible amphiphilic compounds to an art-recognized model for plaque rupture and superficial erosion and determine their effect *in vivo*. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), a single embodiment may provide broad enablement in cases involving predictable factors such as mechanical or electrical elements, but more will be required in cases that involve unpredictable factors such as most chemical reactions and physiological activity. See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991).

Due to the large quantity of experimentation necessary to administer all possible amphiphilic compounds to a subject and determine their effect *in vivo (*particularly to inhibit all forms/ types of necrosis and to inhibit plaque rupture or superficial erosion); the lack of direction/guidance presented in the specification regarding the same; the absence of working examples directed to the same; the complex nature of the invention; the state of the art which discloses the structural and differences among amphiphilic compounds and the lack of appropriate models for plaque rupture and erosion (see Goodman and Gilman's and Lutgens et al., above); and the unpredictability of the administration of all possible amphiphilic compounds,

undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

7. Claims 1-2, 13-14, and 27 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims are directed to a method for inhibiting macrophage death in a subject having, or at increased risk for developing, cardiovascular disease which comprises administering to the subject an effective amount of an amphiphilic compound which inhibits the intracellular transport of cholesterol within cell, so as to thereby inhibit macrophage death in the subject. The claims also recite methods of inhibiting atherosclerotic lesional complications in a subject and for inhibiting necrosis, plaque rupture and/or superficial erosion in a subject by administering an effective amount of an amphiphilic compound.

The specification of the instant application teaches that the term "amphiphilic compounds" includes, without limitation, "compounds which inhibit cholesterol esterification (e.g., steroids such as progesterone), hydrophobic amines, phenothiazines, ionophores, cytochalasins, lysophosphatides such as lysophosphatidylcholine, lysophosphatidylserine and lysophosphatidylethanolamine, colchicine, nigericin, chloroquine, chlorpromazine, trifluoperazine, monensin and amphipathic amines such as imipramine and UI8666A" (pg 26, lines 29-33; pg 27, lines 1-5). Additionally, the specification only teaches methods for

examining inhibition of intracellular transport of cholesterol in macrophages. That state of the art is such that cholesterol is present in all eukaryotic cell membranes (Alberts et al. Molecular Biology of the Cell, New York: Garland Publishing, 1994; pg 480-482). Thus, the brief description in the specification of a few examples of amphiphilic compounds and one example of a type of cell is not adequate written description of an entire genus of methods of using a genus of amphiphilic compounds that inhibit intracellular transport of cholesterol in a genus of cells. To provide adequate written description and evidence of possession of a claimed genus, the specification must provide sufficient distinguishing identifying characteristics of the genus. The factors to be considered include disclosure of compete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof. However, in this case, the specification has not shown a relationship between the structure and function of the claimed genus of amphiphilic compounds and cells.

Vas-Cath Inc. v. Mahurkar, 19USPQ2d 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the 'written description' inquiry, *whatever is now claimed"* (See page 1117). The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed" (See *Vas-Cath* at page 1116).

The skilled artisan cannot envision the amphiphilic compounds and cell types of the encompassed methods, and therefore conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the method. Adequate written description

requires more than a mere statement that it is part of the invention. See *Fiers v. Revel*, 25 USPQ2d 1601 at 1606 (CAFC 1993) and *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016.

One cannot describe what one has not conceived. See *Fiddes v. Baird*, 30 USPQ2d 1481 at 1483. In *Fiddes*, claims directed to mammalian FGF's were found to be unpatentable due to lack of written description for that broad class.

Therefore, only methods of utilizing a specific amphiphilic compound and targeted cell type, but not the full breadth of the claim meets the written description provision of 35 U.S.C. §112, first paragraph. Applicant is reminded that *Vas-Cath* makes clear that the written description provision of 35 U.S.C. §112 is severable from its enablement provision (see page 1115).

35 USC § 112, second paragraph

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

8. Claims 27 and 62-64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

9. Claims 27 and 62-64 are indefinite because the elements recited in the claim do not constitute proper Markush groups. The claims are indefinite in the alternative use of "and/or" because it is not clear what controls which of these limitations. See MPEP § 2173.05(h).

10. Claims 27 and 62-64 are rejected as being indefinite due to recitation of the phrase "inhibiting necrosis...in a subject having, or at increased risk for developing, cardiovascular

disease..." (see claim 27, lines 1-3). The target cell/tissue for the inhibition of necrosis and/or the type of necrosis cannot be determined. For example, are the claims referring to inhibition of macrophage necrosis? Inhibition of neuronal necrosis? Inhibition of atherosclerotic lesional necrosis? Inhibition of radiation necrosis? Inhibition of fibrinoid necrosis? Inhibition of coagulative necrosis?

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

11. Claims 1-2, 13-14, and 27 are rejected under 35 U.S.C. 102(b) as being anticipated by Houser et al. (Cardiovacular Pathol 9(6): 317-322, 2000).

Houser et al. teach that hypercholesterolemic male New Zealand white rabbits are administered doses of progesterone (pg 318, col 1). Houser et al. disclose that feeding that rabbits a C-enriched diet for a relatively short period of time results in advanced aortic atherosclerotic plaques that contain foam cell macrophages and smooth muscle cells histologically similar to human atherosclerosis (pg 320, col 1, first paragraph). Houser et al. teach that high doses of 17-hydroxyprogesterone are significantly associated with less aortic plaque load than controls (abstract; pg 320, col 1-2). (It is noted that it is well known in the prior art that progesterone is an amphiphilic compound and that it inhibits intracellular transport of cholesterol (see for example, Lange et al. 1994; Mazzone et al. 1995; Aikawa et al. 1994).

Conclusion

No claims are allowable.

The art made of record and not relied upon is considered pertinent to applicant's disclosure:

Mazzone et al. J Lipid Res 36 : 544-551, 1995 (progesterone blocks intracellular translocation of free cholesterol in macrophages)

Catsimpoolas et al. U.S. Patent 4,921,838 (teach a method for enhancing angiogenesis or vascular perfusion by administration of a non-ionic amphiphilic compounds)

Lange et al. J Biol Chem 269(47) : 29371-29374, 1994 (teaches amphiphilic compounds, including progesterone)

Aikawa et al. Biochim Biophys Acta 1213(2):127-134, 1994 (progesterone blocks intracellular translocation of cholesterol to the endoplasmic reticulum in macrophages)

Reviews of macrophage apoptosis, necrosis and atherosclerosis

Tabas I. Arterioscler Thromb Vasc Biol 25: 2255-2264, 2005

Tabas I. Cell Death Differentiation 11: S12-S16, 2004

Tabas I. Trends Cardiovasc Med 7 : 256-263, 1997

Studies examining cholesterol trafficking and accumulation in macrophages

Feng et al. Proc Natl Acad Sci USA 100(18): 10423-10428, 2003

Feng et al. Nat Cell Biol 5(9) : 781-792, 2003

Feng et al. J Biol Sci 45(8) : 43271-73280, 2002

Leventhal et al. J Biol Chem 279(9): 8084-8092, 2004

Chen et al. J Biol Chem 276(47) : 43564-43569, 2001

Kellner-Weibel et al. Arterioscler Thromb Vasc Biol 18: 423-431, 1998

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Bridget E. Bunner whose telephone number is (571) 272-0881. The examiner can normally be reached on 8:30-4:30 M-F.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on (571) 272-0961. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

BEB
Art Unit 1647
31 January 2007

Bridget E. Bunner

BRIDGET BUNNER
PATENT EXAMINER

Appendix A

MODES OF CELL DEATH

EM Cabana, DVM (CLSU, Phil '84), MVSt (UQ, Aus '91)
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CELL DEATH

Death of cells in the animal body may either occur normally of pathologically. Three distinct modes of cell death are recognised and include ***Necrobiosis***, ***Apoptosis***, and **Necrosis**. The former two processes are considered part of the normal homeostatic mechanism of the body in regulating cell population, renewal of cells, and in "fine tuning" of organs. As cells continually die even in clinically normal animals, these may be hastened in events of disease processes.

NECROBIOSIS

Necrobiosis is programmed cell death, and involves individual or groups of cells upon reaching their life span. The process occurs virtually unnoticed, with the dead cells replaced by the same cell type following cell reproduction (mitosis and meiosis). Necrobiosis occurs without pathologic sequel because cell function is not interrupted.

Necrobiosis occurs in adults as part of normal cell turnover. An example is red blood cell turnover. The process starts when their haemoglobin molecules begin to precipitate and new haemoglobin cannot be synthesised. Then, the marrow to replace the dying red blood cells produces new red blood cells with "fresh" haemoglobin. Necrobiosis also operates in epithelial turnover in the intestines, where cells are continually produced in the crypts. It also occurs in keratinisation of the skin where cells become filled with keratin. Then, the nuclei degenerate, and the cell desquamate from the skin surface.

APOPTOSIS

Apoptosis is a form of cell death and removal involving one cell at a time. Cells programmed for removal undergo "suicidal action". It may occur normally in several processes that include the following:

1) Embryonic morphogenesis - as in deletion of interdigital tissue in developing chicks
2) Metamorphosis - as in deletion of tail in a tadpole as it matures into a frog
3) Mature tissue homeostasis - as in remodeling of uterus after pregnancy

4) Involution of adult tissue - as may occur in the thymus
5) Pathological states - as in cell death following exposure to ionising radiation and radiomimetic cytotoxic drugs, in malignant neoplasms, and cell-mediated immunity.

In apoptosis, cell death involves one cell at a time, and the dead cell fragments are phagocytosed and digested by resident cells (Figure 5). Like necrobiosis, the process occurs virtually unnoticed, and no pathologic sequels occur. In the literature, several names were used in describing apoptotic fragments before the term was coined. This includes "acidophil or Councilman's bodies" in hepatitis, and "ghost cells" in some skin disorders.

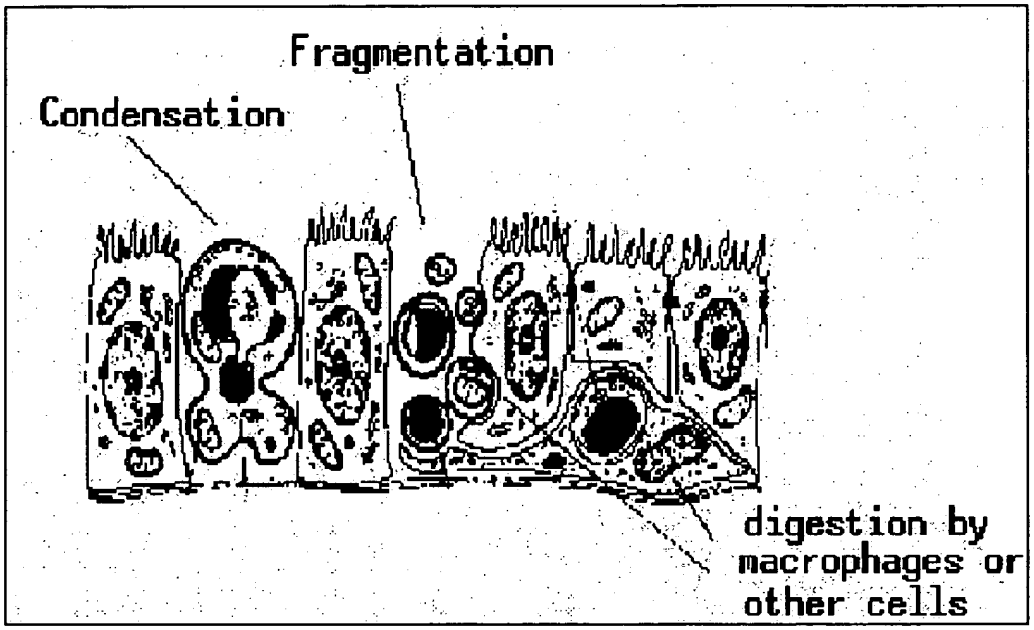

Figure 5. Schematic diagram of Apoptosis

NECROSIS

Necrosis is death of cells or tissues following injury. Cell degeneration may result to necrosis if the injury persists. If it is severe that adaptive responses could no longer operate, and the condition could no longer be reversed, cell death occurs. Unlike the former two modes of cell death, necrosis involves pathological processes and sequel.

Indicators of Necrosis

Necrosis is recognised by:

1) Changes in the nucleus

a) Swelling and clumping of chromatin
b) Pyknosis - condensation of chromatin and shrinkage of the nucleus
c) Kayorrhexis - fragmentation of the nucleus

d) Karyolysis - dissolution of the nucleus by the action of deoxyribonuclease

2) Changes in cytoplasmic staining

a) Positive staining with vital dyes reflecting abnormal membrane permeability
b) Opacification due to denaturation of proteins in the cytoplasm
c) Eosinophilia due to increased affinity to acidic dyes

3) Ultrastructural changes in chronological order as:

a) Margination or progressive loss of nuclear chromatin
b) Focal rupture of the nuclear membrane
c) Breakdown of plasmalemma
d) Development of flocculent densities in the mitochondria

Grossly, necrotic tissue shows the following features:

1) Loss of colour or paleness of tissue
2) Loss of strength in which the necrotic tissue is soft and friable
3) A distinct zone of demarcation is often seen between necrotic and viable tissue.

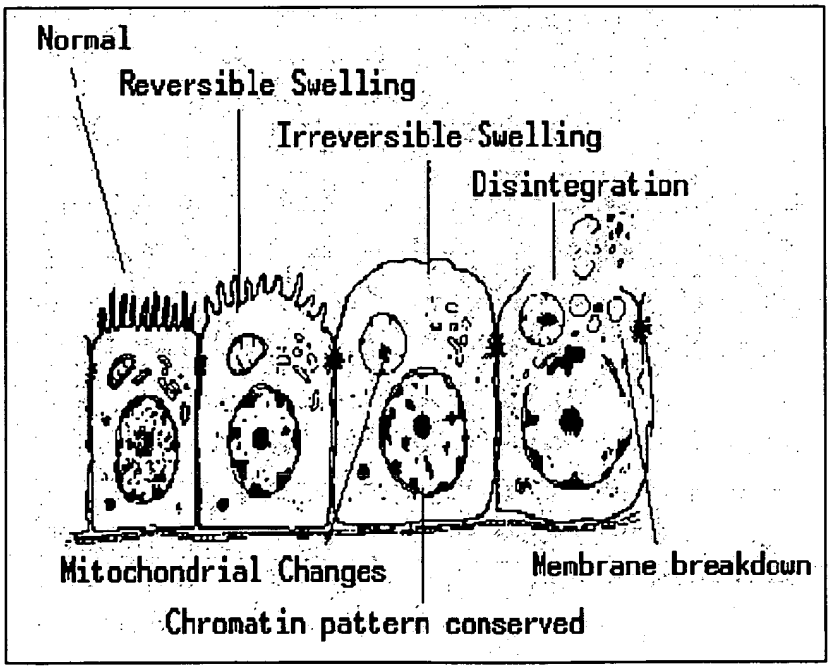

Figure 6. Schematic diagram of necrosis

Biochemical Changes

The process of necrosis involves a series of biochemical changes in cells. The first biochemical indication is the rapid fall in intracellular pH following altered oxygen metabolism in dying cells. These event leads to cessation of oxidative phosphorylation in mitochondria resulting to a further fall in pH. As a result, ATPs are depleted, and the energy dependent Sodium-Potassium pump that regulates the exchange of ions ceases to function. Potassium

ions are released with a consequent influx of sodium ions raising the osmotic pressure inside the cell. This condition draws water towards the cell and results to disruption of organelles, with the consequent release of enzymes stored in the lysosomes. As a result, protein molecules undergo denaturation, and lysis of cell occurs.

At the clinical level, these biochemical changes serve as a source of information that aids in the recognition of certain diseases. The enzymes and denatured protein molecules released from dead cells and tissues find their way into the circulation which could then be identified, measured and the source tissue known. For example, enzymes such as aspartate transaminase (serum glutamic oxaloacetic transaminase or SGOT) and alanine transaminase (serum pyruvic transaminase or SGPT) released from dead liver cells suggests liver disease. The enzyme creatine kinase and denatured protein myoglobin suggest muscle disease.

Basic Types of Necrosis

Two types of necrosis are recognised and are based on the degree of preservation of the architecture of the cells and tissues. These are as follows:

Coagulative necrosis

Coagulative necrosis is characterised by the preservation of cellular and tissue architecture. Microscopically, the nucleus, cytoplasm, and cellular outlines including the arrangements of cells in the necrotic tissue are still intact. This type of necrosis is often difficult to detect grossly, except probably when the affected area is large where subtle changes in tissue colour may be recognised. It usually results from acute disease conditions such as acute toxicity (chemical toxicants or biological toxins) and sudden deprivations in blood supply.

Liquefactive or Lytic necrosis

Rapid enzymatic dissolution of the cell that results in complete destruction is called liquefactive or lytic necrosis (or colliquative necrosis). It is seen in bacterial infections that lead to pus formation in which proteolytic enzymes are released from leucocytes. Pus is the evidence of liquefactive necrosis.

Special Forms of Necrosis

1) Fat Necrosis - occur in two forms: Traumatic Fat Necrosis result from rupture of fat cells because of trauma; Enzymic Fat Necrosis occurs following the enzymic splitting of fat into fatty acid and glycerol by action of lipases (seen in pancreatitis).

2) Zenker Necrosis (Zenker degeneration) - loss of striations in muscles following necrosis (a type of coagulative necrosis in striated muscles).

3) Caseation Necrosis - the presence of friable, cheesy or pasty, amorphous material in necrotic area, usually reserved but not limited to those seen in tuberculous lesions.

4) Fibrinoid Necrosis - a special form of necrosis associated with the accumulation of fibrinoid (see protein overload) in connective tissues and blood vessel walls.

5) Gangrenous Necrosis - necrosis of tissue following deprivation of blood supply, and putrefaction following invasion by saprophytic bacteria. If it is moist, it is called Wet Gangrene. If moisture is not present, it is called Dry Gangrene.

6) Infarct - a form of coagulative necrosis resulting from a sudden deprivation of blood supply (process: infarction, see under haemodynamic changes)

Other Terms Used in Association with Necrosis

1) Malacia - an area of liquefactive necrosis of the nervous tissues. Literally mean "softening"

2) Slough - a piece of necrotic tissue separating from viable tissue. Applied to necrosis of surface epithelia.

3) Ulcer - shallow area of necrosis, applied to epithelial surfaces.

4) Sequestrum - an isolated area of necrosis warded off from viable tissue. Applied to isolated necrosis of bones.

Tissue Reactions to Necrosis

Dead cells and tissues are recognised as foreign and incite tissue reactions. Necrotic tissue may be walled off by proliferating fibrous tissue, incite inflammatory reactions leading to phagocytosis of the necrotic debris, liquefied and drained and the tissue repaired.

The effects of necrosis upon the host vary depending on the type of cells involved, the location of the tissue involved, the number of cells affected, and the rate at which cells are affected. The location and type of cells involved is critical. To illustrate this point, focal necrosis of the liver such as the necrotic tracts caused by migrating nematode larvae may not affect the animal as a whole. In contrast, infarction of the heart may prove fatal to the animal. Similarly, necrosis of neurons would surely cause interference in normal body functions. The rate and number of cells affected also have some bearing on the outcome of necrosis. Slow involvement of cells and tissues may be countered by the process of healing, while rapid death of cells following severe intoxication will produce death in a matter of days. Thus, areas of necrosis encountered during necropsy examinations should be evaluated for its possible significance in the disease process.

Appendix B

4 Cell injury

Overview

Cell injury may be reversible (sublethal) or irreversible (lethal). Many causes may result in reversible injury initially; if severely injured, the cell may be unable to recover and cell death (necrosis or apoptosis) follows.

4.1 Processes involved in cell injury

Learning objectives

At the end of this section you should be able to:

- List the main causes of cell injury and give examples.
- Distinguish reversible from irreversible cell injury.
- Discuss the principal mechanisms of cell injury.
- Describe how the consequences of injury depend on cell-related factors and on cause-related factors.

Causes of cell injury

The causes of both reversible and irreversible cell injury are similar. Many of those listed below may result initially in reversible injury. If the injury is of sufficient severity, the cell reaches a 'point of no return' and irreversible injury culminating in cell death will occur.

Possible causes include:

- hypoxia, e.g. myocardial ischaemia (reduced blood flow to, and therefore oxygenation of, the heart) as a result of coronary artery atherosclerosis
- immunological, e.g. thyroid damage caused by autoantibodies (antibodies produced by the body against its own tissues)
- infection by micro-organisms, e.g. bacterial, viral, fungal infections (such as tuberculous infection of the lung or damage to respiratory mucosa by influenza virus)
- genetic, e.g. Duchenne muscular dystrophy or sickle cell disease
- physical, e.g. radiation (such as sunburn due to UV light damage to the skin), trauma, heat, cold
- chemical, e.g. acid damage to oesophageal mucosa (accidental or deliberate).

Mechanisms of cell injury

The structure and metabolic function of the cell are interdependent. Therefore, although an injurious agent may target a particular aspect of cell structure or function, this will rapidly lead to wide-ranging secondary effects. Recognised mechanisms of cell injury include:

- cell membrane damage
  - —complement-mediated lysis via the membrane attack complex (MAC)
  - —bacterial toxins
  - —free radicals
- mitochondrial damage leading to inadequate aerobic respiration
  - —hypoxia (lack of oxygen)
  - —cyanide poisoning
- ribosomal damage leading to altered protein synthesis
  - —alcohol in liver cells
  - —antibiotics in bacteria
- nuclear damage
  - —viruses
  - —radiation
  - —free radicals.

Free radicals and cell membrane damage

Free radicals are highly reactive atoms which have an unpaired electron in an outer orbital. They can be produced in cells by a variety of processes, including radiation, normal metabolic oxidation reactions and drug metabolism processes. The most important free radicals are derived from oxygen, e.g. superoxide and hydroxyl ions. Free radicals can injure cells by generating chain reactions, producing further free radicals, which cause

cell membrane damage by cross-linking of proteins and by critical alterations of lipids.

Ion transporter function and intracellular calcium
A large proportion of a cell's energy consumption is used by the ion transporter mechanisms ('membrane pumps'). Failure of ATP synthesis (usually because of hypoxia) can result in failure of these mechanisms. Consequently, there is a rise in intracellular calcium and sodium ions and a reduction in intracellular potassium ions. If the endoplasmic reticulum is damaged, sequestered calcium is released resulting in a further increase in intracellular calcium.

Raised intracellular calcium can have a number of effects. It may initiate the caspase cascade (see Section 4.2) causing apoptosis. It can also activate proteases and phospholipases causing further damage to cell cytoskeleton and membranes, and thus contribute to necrosis.

Consequences of cell injury

The consequences of cell injury depend on both the cell and the injurious agent.

Cell features
Certain features of cells make them more vulnerable to serious sequelae of cell injury.

Specialisation Cells that are enzyme rich or have specialised organelles within the cytoplasm may be more vulnerable. The presence of specialised proteins within the cell may make it prone to certain types of injurious agent.

Cell state Cells that have an inadequate supply of oxygen, hormones or growth factors or lack of essential nutrients may be more prone to injury.

Regenerative ability The potential of a cell population to enter the cell cycle and divide is important in the response of tissues to injury. Damaged areas in tissues made up of cells which can divide may be restored to normal, while populations of permanent cells will be incapable of regeneration.

Injury features
In addition, the character of the injury will also affect the severity of the damage.

Type of injury The injury may be ischaemic, toxic, chemical, etc. Different cells will be more susceptible to some injurious agents than others (heart muscle cells are more susceptible to oxygen depletion than connective tissue cells).

Exposure time The length of time of exposure to a toxin or reduced oxygen concentration will affect the chance of a cell surviving the insult. Even relatively resistant cells will be damaged if the duration of exposure is prolonged.

Severity The ability to survive an injury will also depend upon its severity, e.g. is the lack of oxygen partial (hypoxia) or complete (anoxia).

Irreversible cell injury

When does reversible injury become irreversible? The exact 'point of no return' from reversible to irreversible cell injury (leading to cell death) has not yet been defined, although severe mitochondrial damage and cell membrane destruction via free radical generation have been proposed. The light microscopical changes seen in injured cells are well described:

Early changes These are reversible and include:

- cytoplasmic swelling and vacuolation
- mitochondrial and endoplasmic reticulum swelling
- clumping of nuclear chromatin.

Late changes These are irreversible and include:

- densities in mitochondrial matrix
- cell membrane disruption
- nuclear shrinkage (pyknosis)
- nuclear dissolution (karyolysis)
- nuclear break up (karyorrhexis)
- lysosome rupture.

Death of the cell will follow the development of the late morphological changes.

4.2 Cell death

Learning objectives

At the end of this section you should be able to:

- Define autolysis, apoptosis and necrosis.
- Describe the features distinguishing necrosis from apoptosis and give examples of these processes.
- Describe the characteristics of five major types of necrosis.

There are three main forms of cell death: autolysis, apoptosis and necrosis.

Autolysis

Autolysis is the death of cells and tissues after the death of the whole organism. It is also seen when tissue is surgically removed from the organism. The cells are degraded by the post-mortem release of digestive enzymes from lysosomes.

Apoptosis

Apoptosis is also known as programmed cell death. It can occur in normal tissues, for example as a means of regulating the number of cells in a tissue or organ, and during embryological development. It is also seen in pathological processes.

Examples of physiological apoptosis:

- embryogenesis: e.g. formation of digits from the limb buds
- menstrual cycle: endometrial cell loss
- breast feeding: reversal of changes in the lactating breast once breast feeding is finished
- immune cell development: deletion of immune cells (T cells) that may react with the body's own tissues.

Examples of pathological apoptosis:

- tumours: the balance between apoptosis and cell proliferation is disturbed in neoplasia
- atrophy: cell loss in atrophic tissues is by apoptosis (viral illness: e.g. hepatitis—individual hepatocytes can be seen in apoptotic forms)
- AIDS (acquired immune deficiency syndrome): loss of lymphocytes is by apoptosis.

Apoptosis is brought about by a complex system of cell signalling pathways and enzyme-induced events. An important group of enzymes is the caspase family, which acts as an enzyme cascade. As seen in other enzyme cascade reactions, such as the complement system (see Ch. 5), this process serves to amplify the initial apoptotic signal. Some caspases activate other enzymes; others have a direct effect on the structure of the cell by breaking down components of the cytoskeleton. Endonucleases break down DNA into regular fragments at internucleosomal sites, and phospholipases change the configuration of cell membranes.

Morphologically, apoptotic cells shrink and the nucleus condenses. The organelles and nucleus break up, and then the cell breaks into fragments called **apoptotic bodies**. These express ligands on their surface membranes that are recognized by phagocytes, promoting their uptake by phagocytes and, uniquely, neighbouring normal cells (Fig. 7).

In contrast to necrosis, the cell membrane pumps remain viable and continue to function until the terminal stages of the process. Apoptosis does *not* provoke an inflammatory response.

The control of apoptosis is crucial in the process of neoplasia. Some genes involved in cancer formation (e.g. the bcl-2 oncogene) seem to be able to switch off apoptosis, allowing cells to live forever.

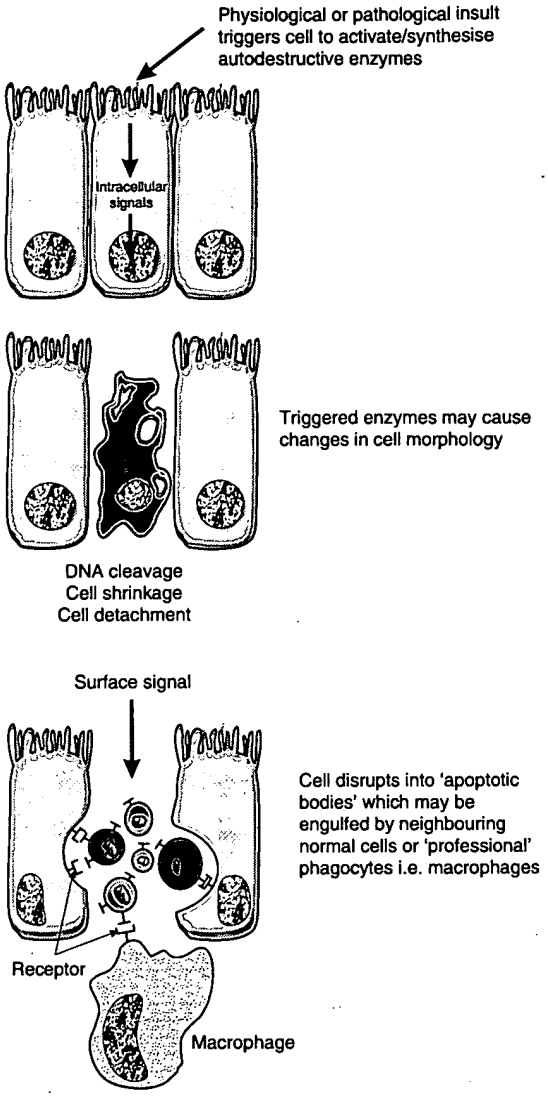

Fig. 7 Apoptosis.

Necrosis

Necrosis is the death of cells in living tissues characterised by the breakdown of cell membranes. It is always pathological. In necrosis, death of a large number of cells in one area occurs, as opposed to the selective cell death of apoptosis (Fig. 8). These changes occur because of digestion and denaturation of cellular proteins, largely by release of hydrolytic enzymes from damaged lysosomes. In fact, the final appearance of the necrotic area will depend on the balance between these two processes. There are several forms of necrosis (see below).

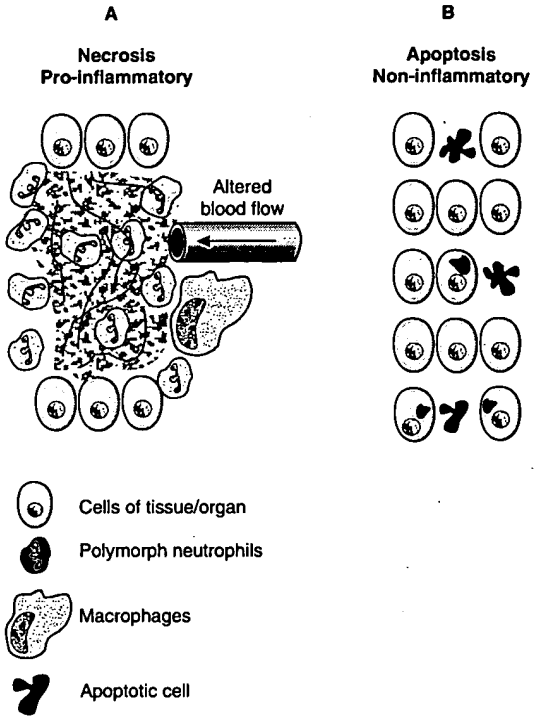

Fig. 8 Diagramatic comparison of necrosis and apoptosis.

The results of cell death include:

- cessation of function of a tissue or organ
- release of cellular enzymes; these can sometimes be detected in the blood and used as markers of the extent or timing of damage to a particular organ, e.g. cardiac enzymes after myocardial infarction
- initiation of the inflammatory response (vital reaction).

Types of necrosis

There are five main types of necrosis (Fig. 9):

- coagulative
- caseous
- liquefaction
- fat
- gangrenous.

Coagulative necrosis Denaturation of intracellular protein (analogous to boiling the white of an egg) leads to the pale firm nature of the tissues affected. The cells show the microscopic features of cell death but the general architecture of the tissue and cell ghosts remain discernible for a short time. Coagulative necrosis is the commonest type, typically seen in, for example, the kidney and heart, and is usually caused by ischaemia.

Caseous necrosis This cell death is characteristic of tuberculosis (TB) and is seen only rarely otherwise. The

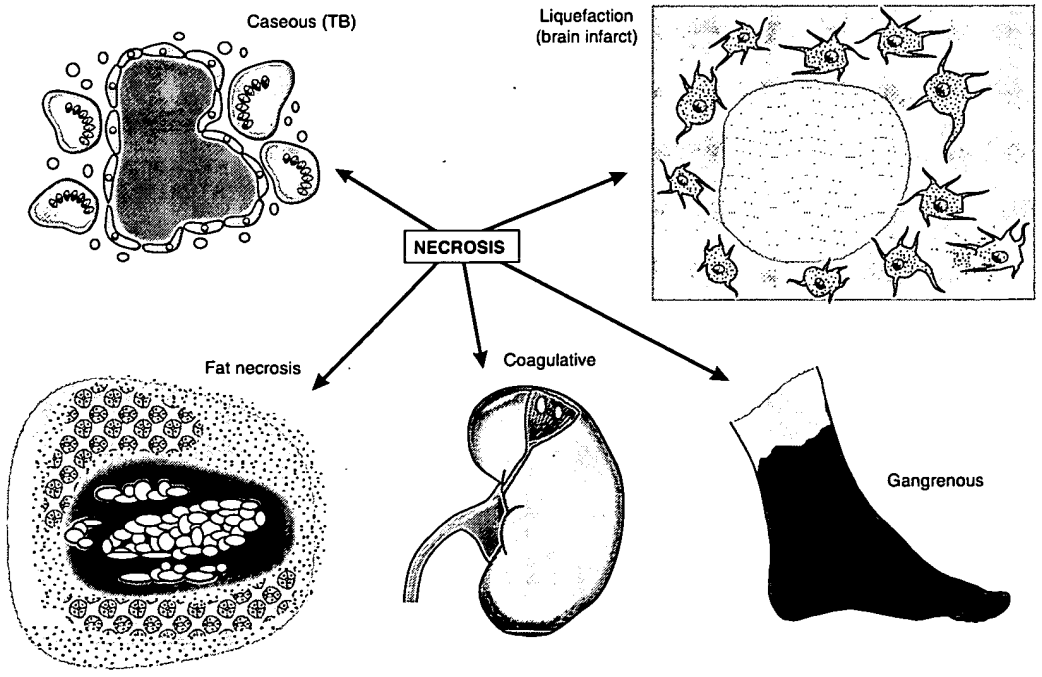

Fig. 9 Types of necrosis.

creamy white appearance of the dead tissue resembles cheese and is probably a result of the accumulation of partly digested waxy lipid cell wall components of the TB organisms. The tissue architecture is completely destroyed.

Liquefaction (colliquative) necrosis This is characterised by tissue softening with destruction of architecture. The result is an accumulation of semi-fluid tissue. It is usually seen in the brain and spinal cord.

Fat necrosis This can result from direct trauma (common in the fatty tissues of the female breast) or enzyme release from the diseased pancreas. Adipocytes rupture and released fat undergoes lipolysis catalysed by lipases. Macrophages ingest the oily material and a giant cell inflammatory reaction may follow (see Ch. 9). Another consequence is the combination of calcium with the released fatty acids (saponification).

Gangrenous necrosis (gangrene) This life-threatening condition occurs when coagulative necrosis of tissues is associated with superadded infection by putrefactive bacteria. These are usually anaerobic Gram-positive *Clostridium* spp. derived from the gut or soil which thrive in conditions of low oxygen tension. Gangrenous tissue is foul smelling and black. The bacteria produce toxins which destroy collagen and enable the infection to spread rapidly; it can become systemic (i.e. reach the bloodstream, septicaemia). If fermentation occurs, gas gangrene ensues. An example is gangrene of the lower limb caused by a poor blood supply and superimposed bacterial infection. This is a life-threatening emergency and the limb should be amputated.

Sometimes, the word gangrene is used to describe the necrotic death of part of a limb when there is little or no infection. In this case, the term 'dry gangrene' is used and the process resembles mummification.

Table 4 Apoptosis versus necrosis

Apoptosis	Necrosis
Membrane integrity preserved	Membranes breached
No inflammation	Inflammatory response
Single cells	Contiguous cells
Active process; requires protein synthesis and consumes ATP	Passive process

The principal differences between apoptosis and necrosis are summarised in Table 4. In many pathological circumstances, both processes may be involved. For example, in myocardial infarction the damage at the centre of the infarct is mainly due to necrosis, but at the periphery (where the hypoxia is less severe) apoptosis may be more important.